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BMB Reports - Manuscript Submission

Manuscript Draft

DOI: 10.5483/BMBRep.2022-0178

Manuscript Number: BMB-22-178

Title: Signaling pathways underlying nitrogen transport and metabolism in plants

Article Type: Mini Review

Keywords: Nitrogen; Plants; N transporter; N signaling; Signaling pathways

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Mini Review

Signaling pathways underlying nitrogen transport and metabolism in plants

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Running title: Nitrogen signaling in plants

Keywords: Nitrogen, Plants, N transporter, N signaling

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ABSTRACT

Nitrogen (N) is an essential macronutrient required for plant growth and crop production. However, N in soil is usually insufficient for plant growth. Thus, chemical N fertilizer has been extensively used to increase crop production. Due to negative effects of N rich fertilizer on the environment, improving N usage has been a major issue in the field of plant science to achieve sustainable production of crops. For that reason, many efforts have been made to elucidate how plants regulate N uptake and utilization according to their surrounding habitat over the last 30 years. Here, we provide recent advances focusing on regulation of N uptake, allocation of N by N transporting system, and signaling pathway controlling N responses in plants.

INTRODUCTION

Nitrogen (N) is one of the macronutrients required for plant growth and development as it is a component of amino acids and plant metabolites (1-3). Amino acids not only function as units of protein, but also function as N donors for many biological compounds including nucleic acids, hormones, and chlorophylls (4-6). Plants uptake N sources in forms of inorganic N (nitrate and ammonium) or organic N (amino acids and peptides) (1, 7). Among them, inorganic N is the major source of N acquisition in plants from the environment (7, 8). However, residual N source in natural conditions is generally insufficient for plants, especially crop plants. To overcome the shortage of N in agricultural fields, additional application of N source in a form of chemical fertilizer has been extensively used to maximize growth and reproduction of plants (9). Plants then absorb nutrients using their own uptake systems. The problem is that plants can only partially absorb fertilizers from the soil.

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Residual fertilizer is released to the environment (10, 11). Fertilizers released often increase active nutrients in the environment, leading to abnormal growth conditions for indigenous organisms. These alterations can cause several environmental problems such as soil acidification and eutrophication. For these reasons, development of plants with improved nutrient use efficiency has been regarded as a strategy to reduce N input and release of N to the environment while maintaining plant performance and productivity (1, 11, 12). To achieve this goal, precise understanding of the mechanisms by which plants utilize N from their habitat is essential. N use efficiency of plants can be determined by major N metabolic processes consisting of N uptake, utilization, and mobilization (1). Here we summarize current our understandings on biological functions of N transporters and N-mediated signaling pathways.

Functions of nitrate transporters and their regulation for N acquisition

To absorb inorganic N forms from soils, plants require nitrate and ammonium transporters expressed in roots. Nitrate acquisition is achieved by two nitrogen uptake systems consisting of a low-affinity transporter system (LATS) and a high-affinity transporter system (HATS) based on their affinity toward N (13, 14). These nitrogen uptake systems involve multiple genes belonging to nitrate transporter 1 (NRT1), nitrate transporter 2 (NRT2), ammonium transporter 1 (AMT1), Ammonium transporter 2 (AMT2) (15, 16).

NRT1 members have been identified as low affinity transporters for nitrate except for AtNTR1.1 whose nitrate affinity is changed by post-translational modification. *AtNRT1.1* was the first plant nitrate transporter identified from Arabidopsis through chlorate resistance screening with T-DNA insertional mutants (17). *AtNRT1.1* was originally identified as a gene

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involved in low-affinity nitrogen transport. Later it was found that *AtNRT1.1* could also function as a high-affinity nitrogen transporter (18). The functional conversion of AtNTR1.1 is controlled by phosphorylation on Thr-101. Dephosphorylated AtNRT1.1 functions as a low-affinity transporter. However, phosphorylation of Thr-101 can changes AtNTR1.1 to be a high-affinity transporter (13, 19). Similarly, MtNRT1.3 was identified as a dual-affinity nitrate transporter from *Medicago truncatula* (20). Functional conversion of AtNRT1.1 by phosphorylation can be further explained by its structural property. AtNRT1.1 can form homodimers through their N-terminal half facing. Phosphorylation of Thr-101 can interfere with AtNRT1.1 dimer formation (21). Under high nitrogen conditions, dephosphorylated AtNRT1.1 can form a homodimer suitable for low-affinity nitrate uptake, while low N-mediated phosphorylation of AtNRT1.1 triggers conversion of AtNRT1.1 to a monomer, switching it to a high-affinity transporter. This regulatory mechanism of AtNRT1.1 allows rapid adaptation of plants to changing nitrate conditions. For successful operation of the system, phosphorylation of AtNRT1.1 has to be actively regulated according to nitrogen conditions. Phosphorylation of AtNRT1.1 is known to be controlled by calcium signaling. Calcium dependent kinase AtCIPK23 can interact with AtCBL9 or AtCBL1, phosphorylating AtNRT1.1 under low-nitrate conditions (19). Based on this observation, it has been proposed that calcium can act as a secondary messenger in N signaling (22, 23). Nitrate treatment can induce rapid accumulation of cytosolic calcium levels. AtNRT1.1 is required for N-mediated calcium wave (23, 24). Suppression of calcium accumulation by calcium channel blockers or phospholipase C inhibitor treatment greatly changed the expression of nitrogen-responsive genes (24). N-mediated calcium wave can induce transcriptional regulation of nitrogen responsive genes involved in N uptake, metabolism, and signaling. Calcium mediated transcriptional regulation is controlled by calcium sensor protein kinases (CPKs) mainly

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through phosphorylation of NIN-LIKE PROTEINs (NLPs) (23). Phosphorylation at Ser-205 in AtNLP7 increases under high nitrate conditions, leading to its nuclear localization and activation of N-responsive gene expression (23). In addition to calcium signaling, ABA signaling also participates in phosphorylation of AtNRT1.1. AtABI2 interacts with AtCIPK and AtCBL1 to dephosphorylate them, thereby interfering with phosphorylation of AtNRT1.1 (25). In addition to nitrate transporter, AtNRT1.1 also mediates N signaling through regulation of *AtNRT2.1* and *AtANR1* expression (26, 27). Due to affinity change and functions in signaling of AtNRT1.1, it has been proposed that AtNRT1.1 can function as a N transceptor (a portmanteau of transporter and receptor). This idea is further supported by the finding that mutation at Pro-492 to Lys can uncouple transporting and sensing activity of AtNRT1.1 (19).

In contrast to NRT1, NRT2 members are known to be high-affinity transporters. Different from NRT1 transporters, NRT2 transporters generally require another component, NAR protein, for their functions (28, 29). In Arabidopsis, all AtNRT2 transporters except AtNRT2.7 can form a complex with AtNAR2.1 (28, 29). Similarly, three OsNRT2s require OsNAR2.1 for acquisition of nitrate in rice (30, 31). C-terminus of NRT2 and the middle region of NAR2 are required for their interactions (32, 33). Specifically, Arg-100 and Asp-109 of OsNAR2.1 are important for its interaction with OsNRT2.3a for plasmamembrane localization and nitrate transport activity (29, 32). Activity of NRT2 is also affected by post-translational modification. AtNRT2.1 remains phosphorylated at Ser-28 under low nitrate conditions. However, it is rapidly dephosphorylated under high nitrate conditions (34, 35). Phosphorylation at Ser-28 stabilizes AtNRT2.1 under N-limited conditions (36). In contrast, Ser-11 of AtNRT2.1 is dephosphorylated under N starvation conditions (35). Similarly, phosphorylation of Ser-501 in AtNRT2.1 can lead to inactivation of its transporting activity

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under high nitrate conditions (37). Phosphorylation of AtNRT2.1 at Ser-501 can be removed by AtCEPD-induced phosphatase (CEPH) under N-starvation conditions (38). AtCEPH mediates CEP dependent long-distance peptide signaling, which is important for root-to-shoot N-starvation signaling (39). Interestingly, the level of AtNRT2.1 protein is not strongly affected by a short-term exposure to high nitrogen conditions (40). These results together indicate that post-translational regulations are important for rapid regulation of high-affinity nitrate transporters in response to high nitrogen conditions **(Figure 1)**.

Function of ammonium transporter and their regulation for N acquisition

The major form of N source for plants is nitrate in dryland soils and ammonium in flooded or acidic soils (41). Thus, plants require AMTs for ammonium acquisition from soils. AMTs consist of 11-12 transmembrane helix regions and a hydrophobic pore for ammonium transport (42, 43). Due to a strong hydrophobicity of the central pore responsible for ammonium transport, how positive charged ammonium can penetrate the hydrophobic pore of AMT remains unclearly. In case of bacterial AmtB, ammonium is first sequestered at the periplasmic face. Ammonium is then deprotonated, yielding hydrogen ion and ammonia. Hydrogen ion and ammonia follow two separated pathways of AmtB to the cytoplasm. Reprotonation then occurs near the cytoplasmic face (43). AMTs widely exist in plants. They can be categorized into two subgroups: AMT1 and AMT2 (44). Arabidopsis has four AtAMT1s and one AtAMT2. AtAMT1;1, the first plant AMT, was identified through yeast complementation assay using a yeast mutant lacking its two ammonium transports (45). Heterologous expression of AtAMT1;1 in oocyte system has revealed that AtAMT1;1 can act as a high-affinity ammonium transporter (46, 47). Roles of AMT in ammonium transport are

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mainly regulated by their tissue specific expression patterns. Under nitrogen deficiency conditions, expression levels of both *AtAMT1;1* and *AtMAT1;3* are up-regulated in outer cell layers of roots and root hairs, suggesting that they are responsible for ammonium acquisition from soils (48). The absorbed ammonium is then further transported by *AtAMT1;2* which is expressed on endothelial cells (49, 50). Different from AtAMT1s, AtAMT2;1 is not directly related to ammonium acquisition from soils. Rather, AtAMT2;1 is related to xylem loading of ammonium. In the presence of ammonium, AtAMT2;1 is mainly expressed in the pericycle. In addition, *atamt2;1* mutants show reduced translocation of ammonium to shoots and reduced ammonium content in the xylem sap (51), suggesting that AtAMT2;1 mainly functions for root-to-shoot ammonium translocation.

Beside transcriptional controls, activity of AMT is further regulated by post-translational control. In AtAMT1;1, Thr-460 is the target for phosphorylation. Substitution of Thr-460 with A in AtAMT1;1, a mimic for dephosphorylated AtAMT1:1, can result in activation of its ammonium transporter activity. In contrast, a phosphorylation mimic version of AtAMT1;1 does not show ammonium transport activity (52). Similarly, transport activities of other AtAMT1s are controlled by phosphorylation (53-55). Similar to NRT, CIPK23 and CBL1 are involved in phosphorylation of AMT. Under toxic ammonium conditions, CIPK23 together with CBL1 can phosphorylate AtAMT1;1 and AtAMT1;2 to inactivate their transporter activities (56). However, mutation of CIPK23 is insufficient to abolish phosphorylation of AMTs. This observation points out that other components are responsible for phosphorylation of AMTs in plants. For example, CIPK15 was identified as a interactor of AtAMT1.1 (57). Another possible candidate for phosphorylation of AMTs has been identified from rice plants (58). OsACTPK1 is a protein kinase belonging to serine/threonine/tyrosine (STY) protein kinase family. Expression level of *OsACTPK1* is changed according to external ammonium

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concentration. In addition, *OsACTPK1* shows overlapping root cell specific expression in the epidermis and exodermis with *OsAMT1;2*. *In vitro* analysis has shown that OsACTPK1 can phosphorylate OsAMT1;2 at Thr-453. Consistent with *in vitro* data, phosphorylation of OsAMT1;2 is reduced in *osactpk1* mutant under sufficient ammonium conditions. AtAMT1;3 has additional positions for phosphorylation, as well as conserved Thr residue (53). Additional phosphorylation of AtAMT1;3 moderately decreases its transporter activity, indicating that phosphorylation at the C-terminal conserved Thr residue acts as a major switch to prevent excess ammonium accumulation, while additional phosphorylation fine tune the activity of AMT to achieve optimal ammonium uptake. In contrast with these regulations, phosphorylation of AtAMT1;1 at Ser-450 residue by CPK32 increases ammonium transporter activity of AtAMT1;1 (59) **(Figure 1)**.

In addition to phosphorylation, AMT activity is regulated by endocytosis depending on external N concentration. Under N deprived conditions, AtAMT1;3-GFP shows long plasma membrane residence time, indicating that AtAMT1;3 can be accumulated in plasma membrane for ammonium uptake. However, AtAMT1;3 forms a speckle under high ammonium conditions. It is then internalized into cytoplasm. The internalization of AtAMT1;3 clusters occurs mainly by clathrin-mediated endocytic pathway (60). The shutdown mechanism of AtAMT1s either by phosphorylation or internalization is important for preventing over-accumulation of ammonium in plants cells known to cause toxicity.

Uptake and allocation of amino acids

In addition to inorganic N, organic N (amino acid and peptides) is also a N source absorbed from the soil by plants (61). Amino acid transporters (AATs) function in organic N

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acquisition and long-distance allocation of amino acids, which are crucial for supporting plant growth, development, and stress responses. AATs can be divided into two families: amino acid/auxin permease (AAAP) family and amino acid-polyamine-organocation (APC) family (62, 63). AAAP family is further divided into amino acid permeases (AAPs), lysine and histidine transporters (LHTs), lysine and histidine transporters (LHTs), γ-aminobutyric acid transporters (GATs), proline transports (ProTs), and indole-3-acetic acid transporters (AUXs). The APC family has cationic amino acid transporters (CATs), amino acid/chloine transporters, and polyamine H+-symporters (PHSs). In addition, Usually Multiple Amino Acids Move In and Out Transporters (UMAMITs), a new class of transporters, has been identified from plants (64, 65). The acquisition of organic N source from the soil is mediated by root-expressed amino acid transporters such as AAP1, AAP5, LHT1, and LHT6 (66-69). In addition to their roles in amino acid acquisition from the soil, AATs can also mediate root-to-shoot allocation (63). AAP2 and AAP6 can mediate xylem-to-phloem amino acid transfer. Phloem loading of amino acids is controlled by multiple AATs (AAP2, AAP3, AAP5, AAP8, CAT1, and CAT6) (63, 70-72). UMAMITs have been reported to be required for amino acid allocation to developing seeds (73). UMAMIT18 present in vascular tissue and developing seeds is required for accumulation of amino acids in developing siliques (73). In addition, UMAMIT14, UMAMIT 28, and UMAMIT29 are involved in allocation of amino acids to the developing embryo (64). AAP8 is also required for amino acid accumulation in developing siliques and seeds (74).

Molecular mechanism of N signaling pathway

N signaling pathway regulates both primary local N responses and systemic N responses.

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Nitrate is a nutrient signal regulating global gene expression in plants. Many genes induced by nitrate treatment are not only directly related to nitrate metabolism, but also involved in other metabolic pathways (amino acid, nucleic acid, other nutrients), hormone signaling, and development (75, 76). Transcriptome analysis of nitrate reductase mutant with impaired nitrate assimilation has shown that nitrate is the primary signal for N responses (77, 78). Nitrate signal is perceived by NRT1.1 nitrate sensor, leading to production of second messengers, which then triggers changes in gene expression (79). As mentioned above, calcium is a strong candidate for nitrate signal transduction. Nitrate treatment can induce rapid increase of cytoplasmic calcium ion through NRT1.1 dependent activation of phospholipase C and inositol phosphate (12, 24). The calcium dependent signaling pathway can transmit N signaling into N-responsive transcription factors through the action of CPKs. CPKs phosphorylates Nin-Line Protein (NLPs), major components for primary N responses in plants (80, 81), to promote their nuclear localization (23). NLP7 is required for both nitrogen sensing and early nitrate dependent signaling (82). Consistent with this idea, majority of CPK-dependent N-responsive genes are overlapped with genes controlled by NLP7. However, some N-responsive genes are regulated through a calcium-independent pathway (23, 24), suggesting that there are additional signaling pathway as well as calcium-dependent pathway. For example, it has been reported that nuclearcytoplasmic movement of NLP7 is controlled by *Homolog of Brassinosteroid enhanced expression2 Interacting with IBH1* (*HBI1*) (83). HBIs are required for activation of antioxidant genes to reduce accumulation of reactive oxygen species (ROS). Disruption of ROS homeostasis either by mutation of *HBI*s or *CATALASE*s attenuates nuclear localization of NLP7. These results indicate that ROS could be another signal that modulates NLP7 dependent N signaling. Contrary to CPKs and HBIs, SnRK1 accelerates cytoplasmic accumulation of NLP7 (84).

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KIN10, the α-catalytic subunit of Snrk1 phosphorylates NLP7 to promote its cytoplasmic localization and degradation. The Snrk1-dependent suppression of NLP7 is required for coordination of carbon and nitrogen metabolism **(Figure 1)**.

Transcriptional network governed by N signaling has been extensively investigated by functional characterization of individual TFs (12, 85, 86). Through the single gene level approaches, many TFs important for nitrate responses, such as ANR1, TGA1, TGA4, NLP6, NLP7, TCP20, LBD37, LBD38, LBD39 and NRG2, have been identified in plants (87-92). They are involved in transcription regulation of various nitrogen responses such as lateral root growth, N uptake, and N assimilation. The first TF identified in N signaling was ANR1, a MADS box gene involved in lateral root elongation (92). Members of the NLP family, in addition to NLP7, are also involved in N signaling. Upregulation of N-inducible gene involved in N transport, assimilation, and metabolic pathways is completely abolished in a *nlp* septuple mutant (*nlp2 nlp4 nlp5 nlp6 nlp7 nlp8 nlp9*). The redundant function of NLPs can be explained by their protein-protein interactions (93). NLP protein form a homo-hetero complex through their PB1 domain. The interaction is required for full activation of target genes (93). In addition, it has been reported that NLP7 forms a complex with *Nitrate Regulatory Gene 2* (*NRG2*) and *TCP20* (89, 90). *NRG2* was identified from forward genetic screening for reduced N responses (90). NRG2 interacts with NLP7 in the nucleus and controls N-mediated expression of N transporters, including NRT1.1 (90). TCP20 and NLP6/7 form heterodimers and bind to adjacent sites in the promoter region of nitrate reductase gene, *NIA1* (89). Interestingly, it has been predicted that *NLP2* can regulate N signaling pathway different from *NLP7* (88). Further investigation is required to determine whether *NLP2* consists new regulatory loop(s) independent of *NLP7*. *TGA1* and *TGA4* have also been identified as key regulatory components in N-mediated root development (87).

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Most of downstream genes controlled by *TGA1* and *TGA4* are involved in N responses. Especially, *TGA1* and *TGA4* regulate nitrate-dependent lateral root development via *NRT2.1* and *NRT2.2* (87). In addition to those positive regulators, negative regulators have also been identified in N signaling. *LBD37-39* are characterized as negative regulators involved in modulation of nitrate-inducible gene expression in a time- and concentration- dependent manner (91). *Interact With Spt6* (*IWS1*) can represses the expression of *NRT2.1* through histone methylation under high N conditions (94). Similarly, *Nitrate-Inducible GARP-type Transcriptional repressor1* (*NIGT1*) act as a negative regulator in N signaling. *NIGT1* expression is positively regulated by nitrate through NLPs. NIGT1 also binds to its own promoter, forming a negative feedback regulation loop. Nitrate-induced *NIGT* can directly represses expression of *NRT2.1* (95). Through this mechanism, *NIGT1* and *NLP*s modulate the expression of *NRT2.1* under given N conditions.

Based on these information and advances in systemic approaches, several attempts have been made to construct N signaling network using machine-learning technology, cell-based TF perturbation analysis, and Y1H analysis (85, 86, 96-98). To identify TFs and their targets in N-mediated root responses, Gaudinier et al. (2018) have performed yeast one-hybrid analysis with 98 promoters and 345 transcription factors involved in N metabolism and responses and constructed a nitrogen-associated metabolism network. The network has confirmed that combinational interactions between multiple TFs are important for regulation of N metabolism and signaling. In addition, several transcription factors (RAV2, ERF107, ARF18, BBX16) involved in hormonal responses are predicted to link N signaling and hormonal regulation of plant growth (86). The advance of TARGET (Transient Assay Reporting Genome-wide Effects of Transcription factors) system has greatly improved our understanding of transcriptional regulatory network. TARGET is a plant cell based temporal

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TF perturbation system based on protoplast transient expression of glucocorticoid receptor (GR)-tagged TF and time course chromatin-immunoprecipitation (ChIP) (99). TARGET technology has been used to identify genome-wide targets of N-responsive TFs (85, 100, 101). Medici et al. (2015) have successfully identified direct targets of *NIGT1* using TARGET. In addition, together with 4-thiouracile labeling of *de novo* transcripts, Para et al. (2014) have found that *bZIP1* regulates early N responsive genes through heat and run transcription. This suggests that TFs can regulate expression of their targets through both transient and stable associations with their promoters. To monitor transient bindings of TFs on their targets, DNA adenine methyltransferase (Dam) can be fused with TF. TF fused with Dam can mark its binding promoter region with adenine methylation (Dam-ID), even if the binding is transient. By coupling ChIP and Dam-ID, Alvarez et al. (2020) have identified both stable and transient targets of NLP7.

Time-based machine learning method has also been applied to construct dynamic regulatory networks underlying N signaling using time course transcriptome profiling (96). Precision of the network has been further confirmed using genome-wide TF-target regulation data of *TGA1*, *HHO*, *HHO6*, and *PHL1*. These networks have isolated 146 novel candidate TFs and their targets involved in N responses (96). Similarly, machine learning method has been used to construct network waking chart for transcriptional dynamics of N signaling in roots (97). Network walking has revealed that *TGA1* is responsible for direct regulation of about 40% of N-responsive genes in roots. Moreover, 49 intermediate TFs connecting *TGA1* to its indirect targets have been found (97).

CONCLUSION

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This review summarizes our current understanding on how plants sense surrounding N status and transmit the information to induce physiological responses. We highlight the importance of post-translational regulation of N transporters for rapid and accurate responses toward changing N conditions, which is important for effective acquisition of N source from soils. N-related transporters have functions not only in N acquisition and signaling, but also in diverse biological processes, including auxin signaling, flowering time regulation, stomatal movements, and plant-pathogen interaction. Further investigation on N transporters and their signaling functions in other processes will expand our understanding of how N participates in the modulation of responses of plants in natural conditions. We also illustrated N signaling mechanism through transcriptional network governed by key N-responsive TFs. As discussed in this review, many efforts have been made to characterize molecular regulation of N responses in plants. Together with classical genetic and biochemical approaches, integrative systemic approaches provide new possible regulatory networks involved resided in N responses. Especially, TARGET-based transcriptome analysis with multiple key signaling components will be useful for investigating dynamic N-mediated transcriptional regulation. In addition, development of data integration tools and modeling system is required to use existed high throughput data for precise prediction of N signaling. In addition, detailed characterization of N-responsive TFs is also required to connect missing link between known key TFs and to ensure their functions are conserved in other plants, especially in crop plants. In addition to local dynamic regulation pathways for N-responses, plants possess systemic nitrate signaling pathways to transmit local N stimuli to distal tissues. Compared with local nitrate signaling, systemic nitrate signaling has been poorly understood due to difficulty of uncoupling local responses from systemic responses. Development of an experimental design that can uncouple systemic responses from local responses will greatly improve our

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understanding of systemic nitrate signaling. A comparative study between nodulating plants and non-nodulating plants is another strategy to elucidate molecular components resided in systemic N signaling.

ACKNOWLEDGEMENTS

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Project No. 2021R1F1A1060339), the New Breeding Technologies Development Program (Project No. PJ01654103), Chonnam National University (Project No.202233830001).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. N uptake and signaling pathway in plants. Plants uptake nitrate (NO_3^-) through NRT transporters. NRT1.1 is dual affinity nitrate transporter, whose affinity is changed by phosphorylation through CIPK23 and CBL1/9. In addition to nitrate uptake, NRT1.1 generates calcium (Ca^{2+}) signaling through Phospholipase c(PLC). Ca^{2+} induces CPK-dependent phosphorylation of NLP6/7 transcription factors, leading to nuclear accumulation of NLP6/7. HBI also accelerates nuclear accumulation of NLP6/7 by reducing cellular reactive oxygen species (ROS) level. Nuclear localized NLP6/7 interact with NRG2 and TCP20 to activate expression of N-responsive genes. NLP2, ANR1, and TGA1/4 act as positive regulator for N-responsive genes. In contrast, LBDs, NIGT1, and IWS1 negatively regulate expression of N-responsive genes. NRT2.1 is a high affinity nitrate transporter, and phosphorylation inactivates NRT2.1 under high N conditions. NRT2.1 phosphorylation was removed by CEPD-induced phosphatase (CEPH) under N starvation condition, activating NRT2.1 dependent nitrate uptake. Ammonium transporter 1;1 (AMT1;1) is responsible for ammonium (NH_4^+) uptake. AMT1;1 is inactivated by phosphorylation through CIPK23 and ACTPK1 under high NH_4^+ conditions to inhibit toxic accumulation of NH_4^+ in cells. The figure was created with Biorender.com

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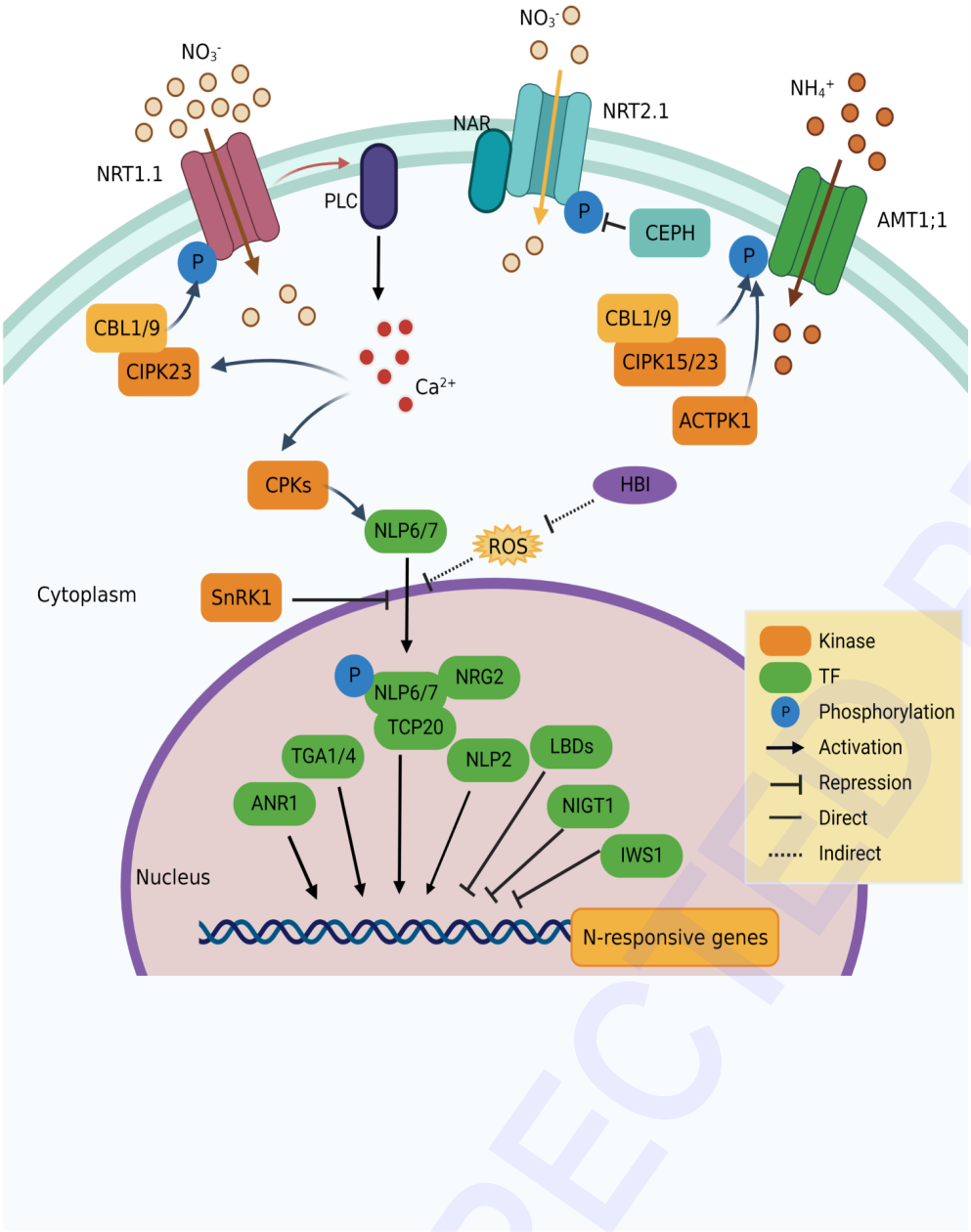

Fig. 1.

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